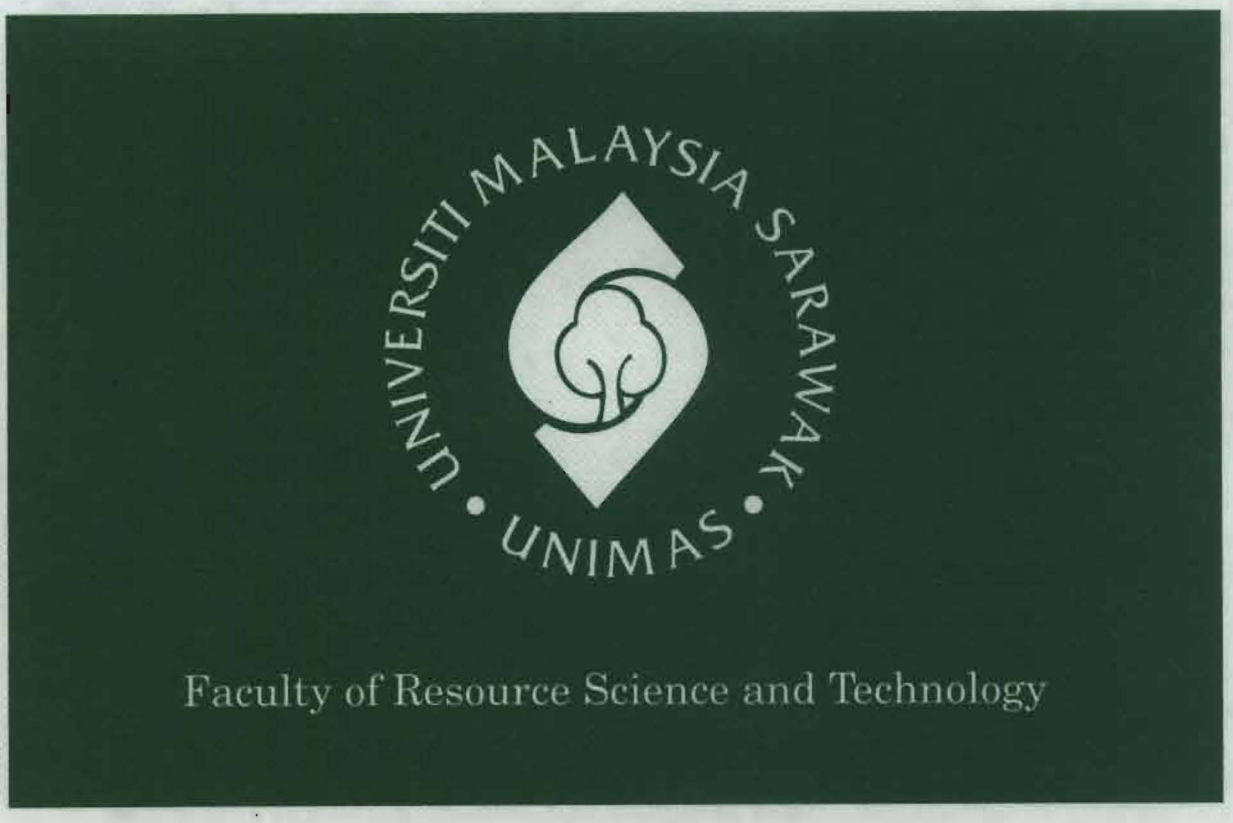

ISOLATION OF cDNA FRAGMENT ENCODING STARCH BRANCHING ENZYME (ISOFORM I) GENE FROM *METROXYLON SAGU BY* RT PCR METHOD

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Bachelor of Science with Honours
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This Project is Submitted in Partial Fulfillment of the Requirements for the Degree of Bachelor of Sciences with Honors
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Statement of Originality

The work described in this Final Year Project, entitled

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has been fully cited to the best of the author's knowledge that of the author except where due reference is made.

24/5/06

Tarikh

Tajuddin Sidek Hairaddin

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ABSTRACT

Isolation and characterization of Starch Branching Enzyme (SBE) isoform I gene was the main objective of this study, where the target was to construct the cDNA from total RNA by using the specific primer that design manually. The RNA was extracted from plant that widely planted in muddy area that is sago palm (*Metroxylon Sagu*). In this study the extracted RNA was reverse-transcribed into cDNA in order to learn the property of the gene that responsible in starch synthesis that encodes for SBE isoform I. The RT PCR technique was used in this study to construct cDNA. Beside that, the comparison method also performs in order to compare which technique will generate cDNA easier. The comparison method that been used was RNase H treatment with the DNA polymerase I. The RNA was successfully extracted by the phenol extraction method with the DNase treatment. The construction of first strand also been achieved by using the RevertAid™ first strand cDNA synthesis kit. Constructions of second strand with the PCR amplification and RNase H treatment were different method that can yield different quantity of cDNA. The cDNA was visualized under UV transilluminator to detect the present of cDNA from the sample.

Keywords: RT PCR, cDNA, *Metroxylon sagu,* RNase H, DNA polymerase I

ABSTRAK

Pengasingan dan Pengelasan gene"Starch Branching Enzyme (SBE) isoform I" merupakan objektif utama dalam kajian ini, yang mana tujuan utamanya ialah untuk menghasilkan cDNA daripada RNA dengan menggunakan spesifik primer yang dihasilkan secara manual. RNA yang diasingkan dalam kajian ini adalah berasal daripada spesies yang biasanya ditanam di kawasan berpaya iaitu pokok sagu (Metroxylon sagu). Dalam kajian ini, RNA yang telah di asingkan akan di terjemahkan kepada cDNA dalam usaha untuk mempelajari latar belakang gene yang dihasilkan oleh kanji dalam pokok ini yang mengkodkan "SBE isoform I". Teknik RT PCR telah digunakan dalam kajian ini untuk membina cDNA. Selain teknik ini, terdapat juga kaedah lain yang digunakan sebagai perbandingan untuk membandingkan teknik yang mana mudah untuk membina cDNA. Kaedah tersebut ialah dengan merawat sampel bebenang pertama dengan RNase H dan juga DNA polymerase. RNA telah berjaya diasingkan daripada daun pokok dengan menggunakan teknik pengekstrakan phenol dan juga rawatan dengan DNase untuk membuang DNA yang terdapat dalam sampel. Pembinaan bebenang pertama juga berjaya dengan bantuan RevertAid™ first strand cDNA synthesis kit. Manakala untuk menghasilkan bebenang kedua, teknik PCR dan rawatan dengan RNase H dan DNA polymerase I yang merupakan dua teknik yang berbeza dalam menghasilkan jumlah cDNA yang berbeza digunakan. cDNA tersebut kemudian diperhatikan di bawah cahaya UV untuk menentukan kehadiran cDNA

***Kata kunci**: RT PCR, cDNA, Metroxylon sagu, RNase H, DNA polymerase I*

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LIST OF ABBREVIATIONS

RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
RT PCR	Reverse transcriptase Polymerase Chain Reaction
SBE	Starch Branching Enzyme
UV	Ultra Violet
Tm	Annealing temperature
LiCl	Lithium Chloride

CHAPTER 1

INTRODUCTION

The genus of *Metroxylon* is found from Thailand, Peninsular Malaysia and Indonesia, to Micronesia, Fiji and Samoa. The palm is generally found in the low altitudes swamp area from 17°S to 15-16°N latitude (McClatchey *et al.*, 2004). There are many different species of *Metroxylon* distributed all over the world. For example, *M. amicarum* is found in the Caroline Islands, *M. paulcoxii* is found in Western Samoa whether *M. sagu* is believed has been originated from Papua New Guinea, New Britain and Molucca Islands (Flach, 1997). Because of the economics importance, the *M. sagu* (sago palm) have been widely distributed towards other country such as Malaysia, Indonesia and Thailand.

In Malaysia, sago palm mainly planted in Sarawak whereby the areas of this cultivation reach up to 25 tonne of starch per hectares. According to Flach (1997), these sago palms are covering more than 2 million hectares. Sago palm is known as one of the oldest tropical plants used by mankind in producing sago starch (Manan *et al.*, 2001). Under a good condition, sago can yield about 15 – 25 mt of air-dried starch per hectares after about 8 years growth cycle (McClatchey *et al.*, 2004). Sago palm consists mainly of energy-living starch with very little protein or minimal content. *Metroxylon sagu* is the main sources of sago starch among palms species. Sago can produce larger amount of starch compare to other sources such as cereals and rice. It can produced starch up to about 250 kg per palm (Gordon, 1999).

Starch is a valuable industrial raw material (Swinkels, 1992) with world wide production of over 40 million tonnes and are predicted to reached about 60 million tonnes for the 2005 (Gordon, 1999). Industrial starch normally being produce by maize, wheat and potato. In order to fulfill the demand of starch, both primary and secondary sources need to study so that the further step can be taken to increase the starch production.

The studies of sago on how to increase the yield of starch production including the propagation, and utilization have been studied for over 20 years ago (Flach and Schuiling, 1991). Modernization has improved the study of sago because people had realized the importance of sago not only in industry but also in medical uses and healthy products. Because of that this study has been done in order to learn more about the specific gene that encoding for Starch Branching Enzyme (SBE) isoform I. The construction of cDNA from *M. sagu* has contributes a lot in continuous study of sago. cDNA was chosen because of it do not have any 'junk' gene or intron that do not encode of any protein.

CHAPTER 2

LITERATURE REVIEW

2.1. Sago palm

The sago palm belongs to the *Lepidocaryoid* subfamily of the *Arecaceae* (*Palmeae*). The scientific name of sago palm is *Metroxylon sagu* Rottboell. Whether the common name that been called by the people is different in different country. For example, in Malaysia they called it rumbia or pohon sagu. But in Solomon Islands they used to call it as ambasao.

The palms are monoecious that is having both male and female flowers on the same plant (McClatchey *et al.*, 2004). Sago palms trunk may reach 10 meters to 12 meter in height and 30 centimeter to 60 centimeter in diameters with basal suckers for suckering (soboliferous) perennial. The leaf was in simple arrangement of pinnate around their trunk and the length is 5 meter to 6 meter in average (Saidin, 1993) with about 50 pairs of leaflets 60 centimeter to 180 centimeter long and approximately 5 centimeters wide (Flach, 1997).

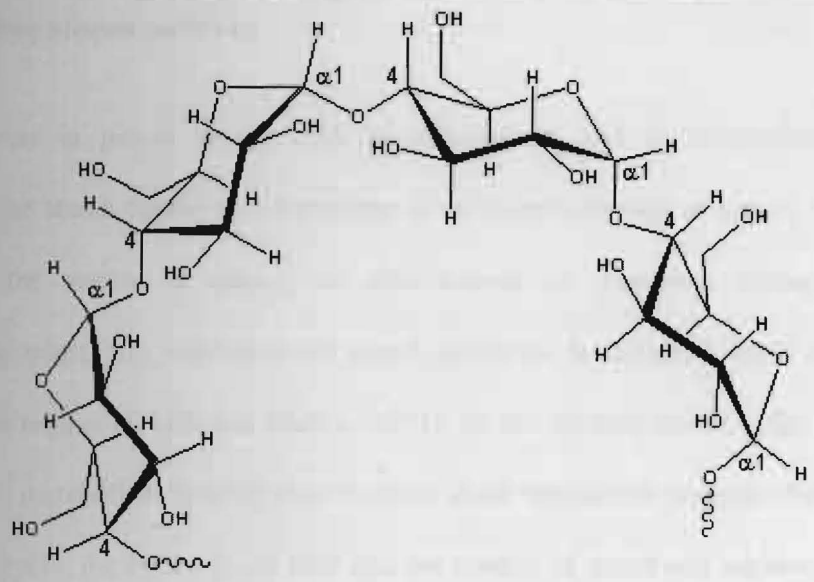
The sago palm is a once-flowering (hepaxantic) that are borne spirally in pairs on the tertiary axis (Flach, 1997; McClatchey *et al.*, 2004). The flowers are bisexual and it will become sago seeds when matured. Pollination take place for about two years and after the pollination the plant dies. This are called monocarpic, that is they fruit and flower once and then die. The life cycle of Sago palm are ranging from 8 to 17 years.

In Malaysia, sago cultivation was mainly concentrated in the state of Sarawak where there are about 1.4 million hectares of peat soil suitable for cultivation of this crop. The cultivation may reach up to 25 tones of starch per hectares (Danjaji *et al.*, 2001). Sago was also known starch crop that have great potential then other crop. The yield of sago towards any other crops such as cassava and maize are 2000-3000 kg/HaYr, 1000 kg/HaYr in maize and 2000 kg/HaYr in cassava (Ahmad *et al.*, 1999).

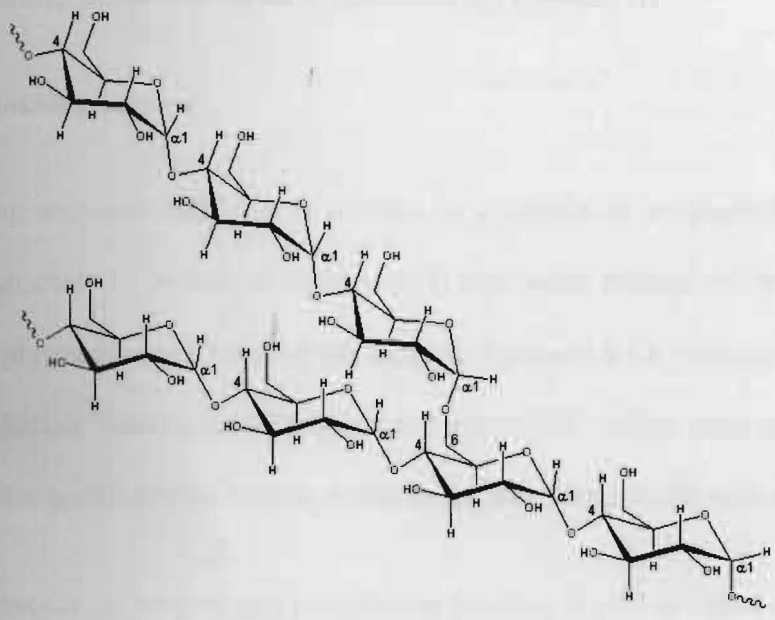
2.2. Composition of starch in plant

Starch is the most abundant storage carbohydrate of higher plant. Plant tubers and seed endosperm provides the major storage place where carbohydrate reserves (as starch) is stored as granules (Buléon *et al.*, 1998). The starch of mature storage organs composed of two classes of glucose polymer namely amylase (a linear chain of α (1-4)-linked α-D-glucose units) and amylopectin (a branched form of amylase cross-linked by α (1-6) bonds). The ratio of amylase to amylopetin in differs according to type of species and also between cultivars within the same species (Shannon and Garwood, 1984). For example in maize, amylomaizes contain over 50% amylose whereas 'waxy' maize has almost none (~3%) (Shannon and Garwood, 1984).

Other than genetic factors, the composition of starch can also be affected by developmental stages. For example, the amylase content of starch increases from 9 to 27% between 8 and 28 days post-anthesis in maize endosperm (Tsai *et al.*, 1974). The molecular structure of amylase and amylopectin are shown in Figure 1.



Representative partial structure of amylase



Representative partial structure of amylopectin

Figure 1: Molecular structures of amylase and amylopectin

2.3. Starch biosynthesis pathway

Starch synthesis in plants occurs both in chloroplasts and in amyloplasts. In the chloroplasts, the starch occurs as a transitory or short-term storage of starch, whether in amyloplasts, the starch is reserve or also known as long-term storage. In the photosynthetic plant, the regulation of starch synthesis is different from those non-photosynthetic organs (Smith and Martin, 1993). In the photosynthetic cells, the whole photosynthetic metabolism is integrated to allow close interaction between the operation of the Calvin cycle, the synthesis of ATP and the control of starch and sucrose synthesis. Whether in storage organ, the cells receive their carbon source as sucrose. The regulation of starch biosynthesis in the chloroplast is illustrated in Appendix III.

2.4. Starch branching enzyme

Starch branching enzymes (SBEs) was involve in synthesis of amylopectin where it create the branch chain by hydrolyze of an α (1,4) glycosidic linkage and subsequent it with an α (1,6) glycosidic bond between the cleaved chain and a C6 hydroxyl group of a α(1,4)glucan. Multiple isoforms of SBE have been reported in various plant species, such as maize, pea, rice, potato, wheat, barley, Arabidopsis, and sorghum (Morell *et al.*, 1997).

The SBE isoforms can be divided into two distinct families, A and B, based on sequence homology (Burton *et al.*, 1995; Safford, 1998). Members of the two classes display distinct enzymatic properties such as substrate preferences and chain transfer patterns. This indicated that both class SBEs may have a different role in starch metabolism. All three isoforms have molecular weights of about 80 kDA (Boyer & Preiss, 1979). Beside

that there are also isoforms from pea embryo, scientifically there are only two isoforms of SBE have been purified that differ in both kinetic and physical properties.

2.5. Reverse transcription-polymerase chain reaction (RT- PCR)

RT-PCR is the most sensitive technique used to detect mRNA and the quantization is available (Sambrook and Russell, 2001). It can be used to quantify from much smaller samples. RT-PCR is actually a modification of basic PCR that used thermostable DNA polymerases. In order to study the RNA using basic PCR, it must be reverse transcribe into cDNA using RT enzymes to provide necessary DNA templates for the thermostable polymerases. This process is called reverse transcription where the RT-PCR name came about.

Generally, Avian myeloblastosis virus (AMV) or Molonery murine leukemia virus (M-MLV or MuLV) reverse transcriptases are used to produce DNA copy from RNA templates using either random primer, oligo (dT) primer or a sequence-specific primer (Ausubel *et al.,* 1999). The basic PCR reaction is carried out after the initial reverse transcription step has produced the cDNA templates. The starting RNA templates can be used total RNA or poly (A) + tail RNA. This project will be using the total RNA as templates for starting the RT-PCR.

2.6. Objective

The main objective of this research is to screen the gene encoding starch branching enzyme (isoforms I) from the cDNA sago that is obtained from the PCR technique and then characterization of the amino acid sequence using computer software. Before screening by PCR can be done, the aim of this project is to construct the primers that are specific to SBE (I) based on sequence of SBE from other plant such as from rice, maize and potato. This research also try to obtain the right sequence using the primer that been construct specifically.

CHAPTER 3

MATERIALS AND METHOD

3.1. Plant materials

Sago leaves for RNA isolation were obtain from 3 to 4 year-old sago palms grown in earthenware pots in the Green House of Faculty of Resource Science and Technology University Malaysia Sarawak. Matured leaves were used for RNA isolation. About 2.0 gm of leaves have been used to yield about 1.0 µL total RNA.

3.2. Chemicals, reagents and enzymes

The chemicals and reagents used were classified in analytical grade, molecular biology grade, biotechnology grade or ultra pure grade that was obtained from Amresco and Sigma. Whether agarose gel were obtained from Promega. The DNA and RNA modifying enzymes such as *taq* polymerase, first strand synthesis kits and DNA Polymerase I were purchased from Fermentas. Others such as RNase-free DNase were provided by Promega. PCR primers were designed manually and constructed by 1st Base Company.

3.3. Basic precautions in laboratory

All the reusable glassware, spatulas, mortars and pestles, centrifuge tubes and others should be treated correctly before and after being used. The apparatus were washed with detergent and then rinsed thoroughly by the tap water and then with the distil water and finally sterilizing it by autoclave at 121°C for 15 minutes.

3.4. Isolation of nucleic acids

3.4.1. Isolation of total RNA from fresh sago leaves

Total RNA will be extracted using the method used by Hussain (2002). In this method LiCl were used in order to remove the DNA that may present in the extraction solution. Whereas Phenol/chloroform/isoamyl was used in order to separate between RNA and Protein from the leaves. Matured leaves firstly were cut into small pieces about 1 cm^2 and then grounded into fine powder in liquid nitrogen by mortar and pestle to yield about 1.5 g powder of sago leaves. 15 mL RNA extraction buffer (150 mM LiCl, 50 mM Tris pH 9.0, 5 mM EDTA and 5% w/v SDS) was added to the sample and vortexed. 15 mL of phenol/chloroform/isoamyl (25:24:1) was then added to the solution and vortexed. After that, the mixture was centrifuge at 6000 rpm for one minute to separate the phase and then the aqueous was removed into the new tube. The phenol/chloroform/isoamyl was performed three times and each times the aqueous is separate into new tube. After that the aqueous solution were added with 15 mL of chloroform and then followed by centrifugation at 8000 rpm for one minute. This step was repeated one more time.

The aqueous solution (containing the RNA) was then being precipitated by addition of 3.3 mL 8 M LiCL to give a final concentration of 2 M LiCl for precipitation of RNA. The extract will be incubated overnight at 4 °C and then centrifuged for 30 minutes at 10, 000 rpm. The RNA pellet will be resuspended in 400 µL of sterile dH_2O. The RNA was precipitated again in 1.0 mL of 100% (v/v) ethanol and 40 µL of 3 M Sodium acetate and put on ice for 20 minutes. The precipitate RNA was centrifuged at 18, 000 rpm for 30 minutes followed by washing twice with 70% v/v ethanol. Total RNA was air-dried and

resuspended in 100 µL of TE buffer (10 mM Tris-HCl pH 8.0, 1.0 mM EDTA) and was then been stored at -80 °C for prior use.

3.4.2 Treatment of DNA by RQ 1 RNase – free DNase kit

RQ I RNase – Free DNase kit were used in the construction of cDNA in order to remove DNA that might present during the RNA extraction process. The DNA present will interfere during RNA process. Because of that DNase treatment is necessary so that when the amplification process, all the PCR reaction will be used to amplify RNA.

The digestion mixture was prepared as followed in the microcentrifuge tube:

RNA in TE Buffer	5 µL
RQ I RNase-free DNase 10x Buffer	1 µL
RQ I RNase-free DNase	1 u/µg
Nuclease-free water to final volume of	10 µL

The mixture was mixed gently by tapping the tube and spun down in microcentrifuge for about 5 seconds. The mixture was then immediately incubated at 37°C for 30 minutes in the water bath. The mixture was then, added with 1µL of RQ I DNase stop solution in order to terminate the reaction. Again the mixture was spun down to mix it. The reaction was then stop by the heat at 65°C for ten minutes in the water bath. This step will lead to the inactivation of DNase enzyme.

3.5. Analysis of nucleic acids

3.5.1. Quantification of RNA by UV spectrophotometric analysis

Five µL of total RNA (treated with DNase) was diluted in 495 µL of sterile dH_20 (1:100 dilutions). The absorbance readings were taken at wavelength 260nm and 280nm. The ratios of A_{260} to A_{280} values were obtained to check the purity of the RNA preparations. Pure nucleic acids have a ratio of A_{260} to A_{280} values range from 1.8 to 2.0.

3.5.1. Qualitative estimation of RNA by agarose gel electrophoresis

The size of the RNA extracted can be checked by the observation through the sharpness of the ribosomal RNA band in the agarose gel. Beside that, the size of the RNA sequences can be estimate based on the ladder that being used. The agarose gel electrophoresis without adding formaldehyde is sufficient to judge the present and also the quality of total RNA preparation. The gel running tank, gel casting tray and the comb were first washed thoroughly with detergent and then rinsed with DEPC-treated RNase free water.

One percent of agarose gel was prepared by melting the mixture of 1.0 gm agarose (promega, ultra pure grade) in 100 mL of 1X TBE, pH 7.8 in side the microwave oven for about 2 minutes. Once the agarose stared boiling, the oven was turn off and the gel were taken out to lower the temperature. When the temperature decreased to about 50°C, then the agarose was poured into the gel casting tray which already contain a comb.

After the gel hardens, 1X TBE running buffer was added into the gel running tank until the gel fully submerged. Five µL of RNA sample mixed with 1µL of 1X Loading Dye Solution (0.25% bromophenol blue) were then loaded into the well. Before that to know the size of the RNA band we use the DNA marker that obtained from fermentas. The marker that we used was GeneRuler^{TM} 1 Kb DNA ladder were also added with loading dye to observe the movement of the RNA. The gel electrophoresis was carried out at 100 V for an hour. The gel was then visualized under a UV transilluminator and photo (Polaroid 667 black and white) was taken by using the ULTRA-LUM Polaroid camera.

3.6. Primer design

In order to obtain the starch branching enzyme (SBE) sequences we have done several procedure to design the primer that might by coded for the production of SBE. The primer design basically about 15 to 30bp long and composed of forward primer and reverse primer. Some applications such as Clustal W and sequences that will take from NCBI homepage are required.

The first thing that must be done was finding the plants that produce the same sequence that were needed. After the plants have been identified, the sequence was obtained by browsing the NCBI webpage. For example, SBE sequence, then the webpage will show list of plant that produce or contain SBE and also the sequence. In order to undergo multiple alignments, more species of plant that coded for SBE sequence were obtained. Multiple alignments is a process whereby three or more sequence to be align in order to find the most conserve region from the sample.

The sequences that were obtained from the NCBI were pasted in the Clustal W program using the Fasta format. This step required internet services to get the result. When the result was obtained, we analyzed it and find the most suitable region to become primer. We need to find the forward and the reverse primer in order to complete the PCR process.

The chosen sequences were send to the commercial company in order to construct the primer. TE buffer must be added to the primers before it can be use. One of the reasons why it needs to add TE buffer is to get the sequence that are come in powder.

3.7. Construction of first strand cDNA

3.7.1. RevertAid™ first strand cDNA synthesis kit

First strand cDNA synthesis was carried out by using the RevertAid™ first strand cDNA synthesis kit that provided by Fermentas. This kit was designed for the preparation of full length first strand cDNA from RNA templates which is relies on a genetically engineered version of Moloney Murine Leukimia Virus reverse transcriptase (RevertAid™ M-MuLV RT) with low RNase H activity. Because of the reaction condition of cDNA synthesis and PCR are compatible it will result that the mixture can be added directly to the PCR mixture without prior purification of the cDNA.

The reaction mixture was prepared in a microcentrifuge tube placed on ice as follow:

Total RNA	5 µL
Sequence-specific primer	1 µL
DEPC-treated water	to 12 µL